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Research Article

BIODEGRADATION OF LOW-DENSITY POLYETHYLENE (LDPE) BY *STAPHYLOCOCCUS KLOOSII* M1 ISOLATED FROM CATTLE DUNG

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ABSTRACT: This study aimed to isolate bacteria from cattle dung for the biodegradation of low density polyethylene (LDPE). Cattle dung were collected from the Instructional Livestock Farm Complex (ILFC), Veterinary College, Mhow, Madhya Pradesh. LDPE films were treated with UV rays for 48 hours and used for the study. Three isolates were identified by Gram's staining. Out of three isolates M1, S1 and K4; two were identified as Gram-positive, and 1 was found as Gram-negative bacteria. Screening of isolates for LDPE biodegradation was carried out using a clear zone method. Out of 3 isolates, M1 was identified as LDPE degrading bacteria. LDPE degradation was analysed by gravimetric analysis using the weight loss method, functional group analysis by Fourier transform infrared (FT-IR) spectroscopy and surface analysis by scanning electron microscopy (SEM). After 60 days of incubation with LDPE films isolate M1 showed significant weight loss (8.08 ± 1.03 %). FT-IR analysis showed a decrease (22.64%) in carbonyl index and changes in functional group vibrations indicating LDPE degradation. SEM image examination revealed bacterial colonization, biofilm formation, surface erosions, cracks and pit formation on the LDPE surface which was indicative of the degradation of LDPE film. Further, the identification of isolate M1 was done by BD Phoenix M50 automated identification and susceptibility testing system and 16S rRNA sequencing. Sequencing and phylogenetic analysis of the M1 isolate showed 98.83% similarity with *Staphylococcus kloosii* strain ATCC 43959 chromosomes (Accession No. OR771911.1). Our findings underscore the potential of strain M1 from cattle faeces to efficiently utilize LDPE as a sole carbon source, thereby contributing to its degradation. This is the first report of isolating LDPE-degrading bacteria from cattle faeces. It is crucial to analyse the cattle dung gut microbiota for plastic degradation since this discovery highlights the need for further investigation.

Keywords: *Staphylococcus kloosii*, Cattle dung, Biodegradation, LDPE, Scanning electron microscopy, Fourier transform infrared (FT-IR) spectroscopy.

INTRODUCTION

Polyethylene has become a serious environmental pollutant due to its high non-biodegradability and has gained the attention of several researchers to find a way to degrade it. Large numbers of animals die due to ingestion of plastic material annually. Stray cattle also badly suffer due to the ingestion of plastics. Approximately 1.5 million marine animals died due to blockage of air passage and 1 million birds died due to ingestion of plastics annually [1, 2]. The use of physical and chemical methods to degrade plastic wastes results in the emission of polychlorinated biphenyls, polycyclic aromatic hydrocarbons, dioxins, and furans gases leading to health problems and environmental pollution [3]. Recently, microorganisms have become the focus of interest for environmentally friendly disposal of plastic and polymer-based waste [4]. Plastic biodegradation is a surface erosion process as the enzymes that break down plastics usually act only on the surface. Biodegradation can be enhanced by exposing polyethylene (PE) to UV, thermal or chemical oxidation. Bacteria can also contribute to the process by creating a biofilm on the plastic surface that damages the surface and modifies its functional groups [5, 6, 7].

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Recently isolation of polyethylene-degrading microbes from gut microflora from wax worms and their larvae suggests that gut microbes can also degrade polyethylene [7]. Thus, gut microflora can also be a new resource for developing functional microbial consortia that can degrade polyethylene and other plastics. In our study, we isolated Staphylococcus kloosii stain M1 from cattle dung and assessed its biodegradation capabilities.

MATERIALS AND METHODS

Selection of LDPE

For the biodegradation experiment, LDPE was selected based on the classification of plastic reported by Mariotti and co-workers [8]. Fifty-micron thick LDPE sheet was selected for the biodegradation study which was obtained from computer wrapping. The thickness was measured by a micrometre caliper (Mitutoyo Absolute Digimatic Caliper Series 500, Japan). LDPE was treated with benzene for 30 minutes to remove any plasticizers, colouring agents, or fillers [9], followed by, washing manually with absolute ethanol using cotton wool and rinsed severally with distilled water to remove dirt and other organic particles adhering to the surface and then allowed to air dry for 30 minutes. LDPE sheet was used for the preparation of films and LDPE powder. Further, these LDPE film and powder was used as a sole carbon source for the growth of bacteria.

Preparation of LDPE films and LDPE powder

LDPE was cut into 1.5 x1.5 cm^2 and sterilized as described by Lee and co-workers; Nanda and co-workers; and Yao and co-workers [10, 11, 12]. Subsequently, LDPE films were dried at 60 ºC in a hot air oven for one hour. UV light exposure (365 nm) (UV tech, Cambridge) was given to these LDPE films for 48 hours as described by Lee and co-workers for photo-oxidation [10].

Further, each LDPE films were weighed separately (Denver Instrument PI- 214.3 Analytical Balance 210g). The dried LDPE films were stored in a glass desiccator until further use.

The LDPE powder was prepared as described by Bhatia and co-workers; and Ghosh with some modifications [13, 14]. This LDPE powder was stored at room temperature in a closed container until further use.

Sample collection

One gram of cattle dung was collected from the Instructional Livestock Farm Complex (ILFC), Veterinary College, Mhow, Madhya Pradesh (Latitude 22.5883591, Longitude 75.7831134) in a sterile flask and transported on ice to the laboratory.

Enrichment of LDPE-degrading bacteria

Mineral salt medium (MSM) broth was prepared as described by Nademo *et al.* (2023) with minor modifications [17]. One gram of cattle dung was inoculated in 50 ml MSM broth containing 0.2% LDPE powder and incubated aerobically at 120 rpm for 30 days in a shaker incubator at 37 ºC for the enrichment of LDPE degrading bacteria. Gram staining was performed to confirm bacterial growth in MSM broth.

MSM agar plates were prepared as described by Nademo and co-workers by adding 1.5% agar [17]. After 30 days of enrichment, one ml of enriched broth was inoculated on MSM agar plates containing 1.5x1.5 cm2 LDPE films and 0.2% LDPE powder as the only carbon source for bacterial growth. Plates were incubated aerobically for 60 days at 37ºC. The gross morphological characteristics of the colonies after 60 days were observed and confirmed by Gram staining. Further, these isolates were characterized biochemically. These bacteria were inoculated on nutrient agar and incubated aerobically at 37 ºC for 1-2 days for isolation.

Screening of LDPE degrading bacteria

Isolates were screened for biodegradation of LDPE by the clear zone method as described by Rana and Rana [15, 16, 17]. Bacterial isolates were inoculated on MSM agar containing 0.2% polyethylene glycol and incubated aerobically at 37ºC for 14 days. After 14 days of incubation, plates were stained with 0.1% Coomassie brilliant blue solution and de-stained with de-staining solution (40% (v/v) methanol and 10% (v/v) acetic acid) to visualize a clear zone around the colony. The bacteria producing clear zones in a blue background were considered polyethylene degraders. One isolate was selected for the LDPE degradation experiment.

Identification of LDPE degrading bacteria

LDPE degrading bacteria was confirmed by BD Phoenix M50 automated identification and susceptibility testing system at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Jabalpur, Madhya Pradesh. Further, confirmation of isolate was done based on Sanger sequencing of conserved 16S rRNA gene sequence by sending isolates to Barcode Biosciences, Bangalore, Karnataka, India.

Submission of sequence to National Centre for Biotechnology Information (NCBI) and analysis of sequence

The cured sequences of the open reading frame (orf) were submitted to NCBI and an accession number was

obtained. Based on the nucleic acid sequences of 16S rRNA, a phylogenetic tree was drawn by Mega 11 [18] considering 1,000 bootstrap values.

Experimental setup of biodegradation of LDPE in MSM agar

Eight MSM agar plates were prepared including 4 control plates. Each plate contained 4 LDPE films (LDPE 1-4). Bacterial isolate selected from clear zone analysis was used for experimentation. The bacterial isolate was compared with a standard 0.5 McFarland, and 4 MSM agar plates were inoculated with inoculum. Control MSM plates were uninoculated. One inoculated and one control plate for each incubation period of 15, 30, 45 and 60 days were kept in the incubator and left for 15, 30, 45, and 60 days. The plates were incubated aerobically at 37°C.

Collection of LDPE film after incubation for gravimetric analysis, functional group analysis, and surface analysis

LDPE films numbered 1 to 3 were removed from MSM agar plates after a 15, 30, 45, and 60-day incubation period. The films were then washed with a 2% sodium dodecyl sulfate (SDS) solution for 30 minutes, followed by a 70% ethanol solution for 30 minutes, and then with distilled water for 30 minutes to remove the bacterial biomass. Then the film was dried at 60ºC for 1 hour and weighed for gravimetric analysis [12, 17, 20]. The gravimetric analysis was done by using the following formula- Weight loss (%) = (Initial weight – Final weight/Initial weight) x 100 [17]. Here, the initial weight of LDPE films after being exposed to UV rays and the final weight of LDPE film after incubation, respectively.

The plates were discarded with proper biosafety precautions after taking observations at each incubation interval. LDPE films No. 3 and 4 were processed for the surface degradation and visualization of bacterial attachment and biofilm formation, respectively under scanning electron microscopy as described by Harshvardhan and Jha [20]

The dried LDPE No. 1 of each plate was stored in the laboratory for further use after gravimetric analysis. The dried LDPE No. 2, 3 and 4 after 60 days of incubation from inoculated and uninoculated (control) were sent for functional group analysis by Fourier transform infrared (FT-IR) spectroscopy (Bruker, Germany, 3000 Hyperion Microscope with Vertex 80 FT-IR system), visualization of bacterial growth and biofilm formation and surface erosion, respectively by Field emission scanning electron microscopy (FE-SEM) (FE-SEM Supra 55, Carl Zeiss, Germany) to Sophisticated Instrumentation Centre (SIC), Indian Institute of Technology (IIT), Indore, M.P. India after gravimetric analysis; and compared results with their respective controls.

A decrease in the weight of LDPE film in gravimetric analysis, the presence of bacteria and biofilm, holes, cracks, groove formation and surface erosions by surface analysis and change in functional groups, shift of functional groups, new peaks formation, or alterations in existing peaks, an increase or decrease in carbonyl index were indicative for microbial activity and LDPE degradation.

RESULTS AND DISCUSSION

MSM agar was used for experimenting with biodegradation of LDPE. It does not contain any carbon or energy source for the growth of bacteria [21]. In this experiment, UV-treated LDPE was added as a sole carbon and energy source for bacterial growth. UV rays are required for the Photooxidation of LDPE that enables function group and carbon to bacteria for growth. UV ray treatment of LDPE enhances the availability of carbon for the growth of bacteria from LDPE [22].

Isolation of LDPE degrading bacteria

After enrichment and inoculation of enriched broth into MSM agar for 60 days, 3 different colonies were observed on the surface of LDPE films named M1, S1 and K4. Out of these three colonies, two were Gram-positive and 1 was found Gram-negative bacteria. Details of bacterial isolates are presented in Table 1.

Table 1. Details of bacterial isolates.

Name of Isolate	Colony characteristics	Gram Staining Result	Catalase test	Oxidase test	Indole test	Methyl Red test	Voges – Proskauer test	Citrate Utilization test
M1	Yellow colonies	Gram positive cocci	+	-	-	-	-	+
S1	White pinpoint colonies	Gram positive cocci	+	+	+	+	-	+
K4	White pinpoint colonies	Gram negative rods	+	-	-	-	-	+

Table 2. Mean weight loss of LDPE films by *S. kloosii* M1 in MSM broth at different incubation periods.

S. No.	Incubation period (Days)	Initial weight (mg)	Final weight (mg)	Weight loss (mg)	Weight loss (%)
1.	60	70.00±9.07	64.33±8.41	5.66±1.20	8.08^{a}±1.23
2.	45	51.33±5.23	48.66±4.97	2.66±0.33	5.18^{ab}±0.41
3.	30	60.00±1.73	58.00±2.08	2.00±0.57	3.36^{bc}±0.98
4.	15	52.00±1.52	51.00±1.00	1.00±0.57	1.86^{c}±1.06

*The mean weight loss (%) with different superscripts differs significantly ($p<0.05$).

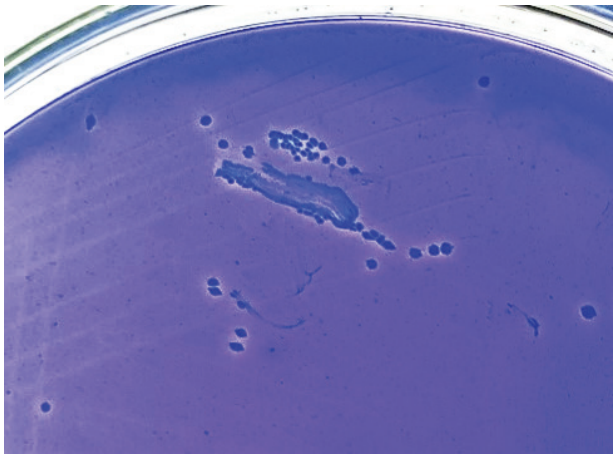

Fig. 1. Clear Zone formation around bacterial isolate M1.

Screening by clear zone analysis

Three isolates M1, S1 and K4 were inoculated for 14 days on MSM agar containing 0.2% PEG. Out of three isolates, M1 was seen as a potential LDPE degrader by observing clear zones around colonies (Fig. 1). Rana and Rana screened 28 isolates and identified 2 isolates *viz*., PDBH1 and PDBM 2 as potential degrader of LDPE [16]. Nademo and their colleagues screened 60 isolated and used 14 isolates for the LDPE degradation experiment [17]. Jain and co-workers screened four isolates and concluded that *Pseudomonas aeruginosa* strain P9 as potential LDPE degrader [33].

Identification of LDPE degrading isolate

The bacterial isolate was identified as *Staphylococcus kloosii* by BD Phoenix M50 automated identification and susceptibility testing system (Identification No. 427630785957) and also confirmed by sequencing of conserved 16S rRNA gene with universal forward and reverse primers *viz*., 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT), respectively. The sequence of isolate M1 showed 98.83% similarity with *Staphylococcus kloosii* strain ATCC 43959 chromosome.

Submission of sequence and sequence analysis

The obtained sequence was cured using bio-edit software with a 100% query sequence of 1545bp. The sequence was submitted to NCBI and OR771911.1 accession no. was obtained.

Further, phylogenetic trees were drawn by Mega 11 [18] based on nucleic acid sequences of 16S rRNA, following the alignment of the *orf* sequences using the Neighbour-joining method considering 1,000 bootstrap values. The BLASTn search result and phylogenetic analysis revealed a high degree of homology between the query sequence and other subject sequences available in the NCBI database (Fig. 2).

On perusal of the literature and to our knowledge, no study was conducted for the isolation of LDPE-degrading bacteria *Staphylococcus kloosii* from cattle dung. However, the isolation of LDPE-degrading bacteria was done by Skariyachan and their colleagues from cow dung [23]. They reported Enterobacter cloacae nov. bt DSCE02 and Pseudomonas aeruginosa nov. bt DSCE-CD03 isolated from cow dung as an LDPE degrader. Gupta and their co-workers isolated an HDPE-degrading bacterial strain from the faecal matter of a cow [24]. The bacterial strain was identified as *Micrococcus luteus* CGK112. Jain and co-workers reported Pseudomonas aeruginosa strain P9 isolated from poultry droppings as LDPE degrade [33].

Determination of LDPE degradation by bacterial isolates

The LDPE degradation was characterized by gravimetric analysis, functional group analysis, by visualization of biofilm and surface erosions.

Gravimetric analysis

A significant increase ($p<0.05$) in mean weight loss (%) of LDPE films was seen in MSM agar media by *Staphylococcus kloosii* M1 at 15, 30, 45, and 60 days of the incubation period. It was found that the degradation of LDPE done by *Staphylococcus kloosii* strain M1 was

increasing significantly with an increase in time interval. The mean weight loss (%) at 15 days of incubation was (1.86±1.06); at 30 days (3.36±0.98); at 45 days (5.18±0.41) and the highest degradation was seen at 60 days of incubation (8.08±1.23). The data shows that *Staphylococcus kloosii* M1 was able to degrade LDPE films actively in MSM agar with an increase in the time interval (Table 2 and Fig. 3). There was no weight loss seen in control groups.

Chatterjee and their colleagues reported catheter-related bacteraemia by isolating *Staphylococcus epidermidis* from a catheter. They showed the importance of biofilm formation by *S. epidermidis* on polymeric materials, used in prosthetic heart valves, stents, and orthopaedic prostheses [25]. Divyalakshmi and Subhashini isolated polythene-degrading bacteria from garbage soil and reported 13.6% weight loss of polyethylene milk covers by *Staphylococcus arlettae* after 30 days of incubation at 37ºC [26]. Vatseldutt and their colleagues reported a 52% weight loss of plastic by *Staphylococcus* species [27]. Jangra and their colleagues isolated 20 bacterial isolates belonging to *Staphylococcus* species and reported their highest plastic degradation ability [28].

Functional group analysis

Functional groups were analysed in the region of 4000-400 cm^{-1} at the resolution of 4 cm^{-1}. The resultant spectrum included a plot of rate transmittance (%) versus wavenumber (cm^{-1}), which was investigated against the respective controls. The FT-IR spectrum of the UV-treated control LDPE film served as a baseline. It represented the LDPE film without any microbial exposure. Peaks in this spectrum corresponded to the inherent vibrational modes of LDPE (Fig. 4)

The FT-IR spectrum of the LDPE film exposed to *Staphylococcus kloosii* M1 reflected changes induced by microbial activity. The analysis was done in a spectrum for shifts, new peaks, or alterations in existing peaks. Specific bands indicated interactions between the bacteria and the LDPE polymer.

After 60 days of incubation, a shift in absorption peak from 2661.62 cm^{-1} to 2638.48 cm^{-1} and 568.97 to 549.68 cm-1 indicated a change in symmetric stretching vibrations of alkyl $–CH_2$ group and vibrational change in C-H bonds, respectively. A shift and decrease in absorption peak of 1608. 54 cm^{-1} to 1575.76 cm^{-1} represents cleavage in the carbonyl bond. A decrease in absorption peak of 2221.87 cm^{-1}, 2117.72 cm^{-1}, 2019.36 cm^{-1}, 1903.64 cm^{-1}, 1812.99 cm^{-1}, 1714.62 cm^{-1}, 1375.17 cm^{-1}, 1301.88 cm^{-1}, 1182.30 cm^{-1}, 1080.08 cm^{-1}, 885.28 cm^{-1}, 773.41 cm^{-1}, 729.05 cm^{-1} and 673.12 cm^{-1} and formation of new peak at 927.71 cm-1, 800.41 cm-1 were seen. These vibrational changes in spectral bands suggested cleavage of functional groups in the LDPE film occurred during the degradation (Fig. 4).

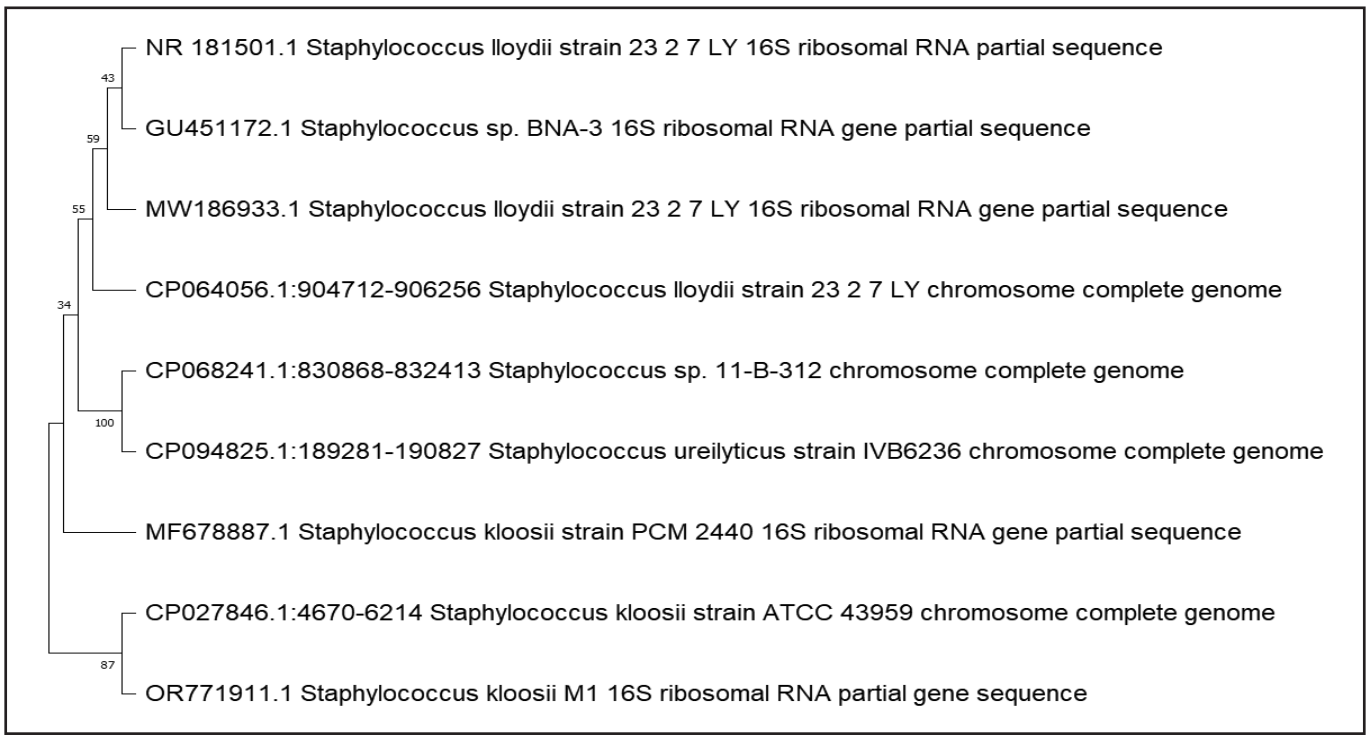
NR 181501.1 Staphylococcus lloydii strain 23 2 7 LY 16S ribosomal RNA partial sequence
43
GU451172.1 Staphylococcus sp. BNA-3 16S ribosomal RNA gene partial sequence
59
MW186933.1 Staphylococcus lloydii strain 23 2 7 LY 16S ribosomal RNA gene partial sequence
55
CP064056.1:904712-906256 Staphylococcus lloydii strain 23 2 7 LY chromosome complete genome
34
CP068241.1:830868-832413 Staphylococcus sp. 11-B-312 chromosome complete genome
100
CP094825.1:189281-190827 Staphylococcus ureilyticus strain IVB6236 chromosome complete genome
MF678887.1 Staphylococcus kloosii strain PCM 2440 16S ribosomal RNA gene partial sequence
CP027846.1:4670-6214 Staphylococcus kloosii strain ATCC 43959 chromosome complete genome
87
OR771911.1 Staphylococcus kloosii M1 16S ribosomal RNA partial gene sequence

Fig. 2. Phylogenetic analysis of *S. kloosii* M1 (Accession No. OR771911.1).

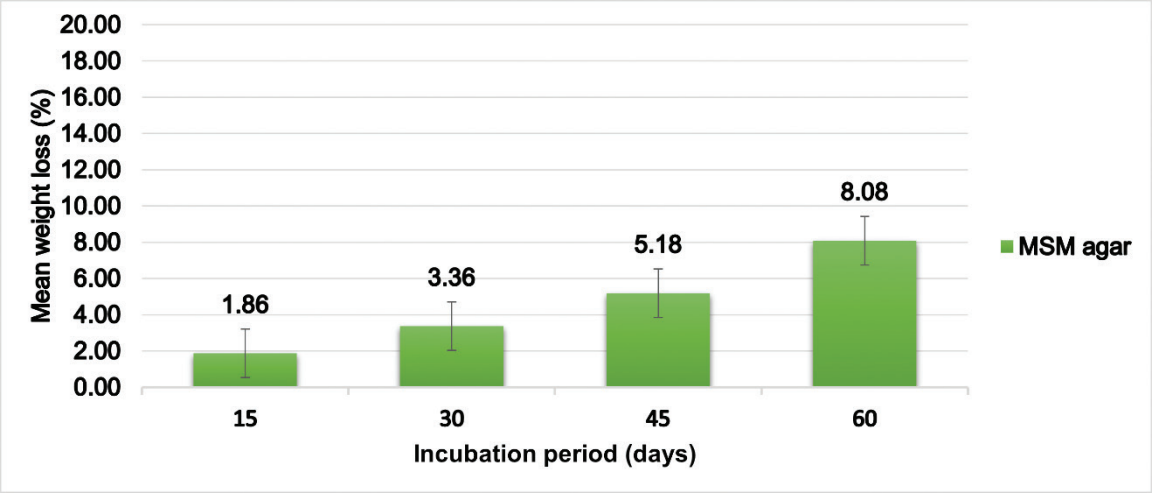

Fig. 3. Mean weight loss (%) of LDPE films by *S. kloosii* M1 in MSM agar at different incubation periods.

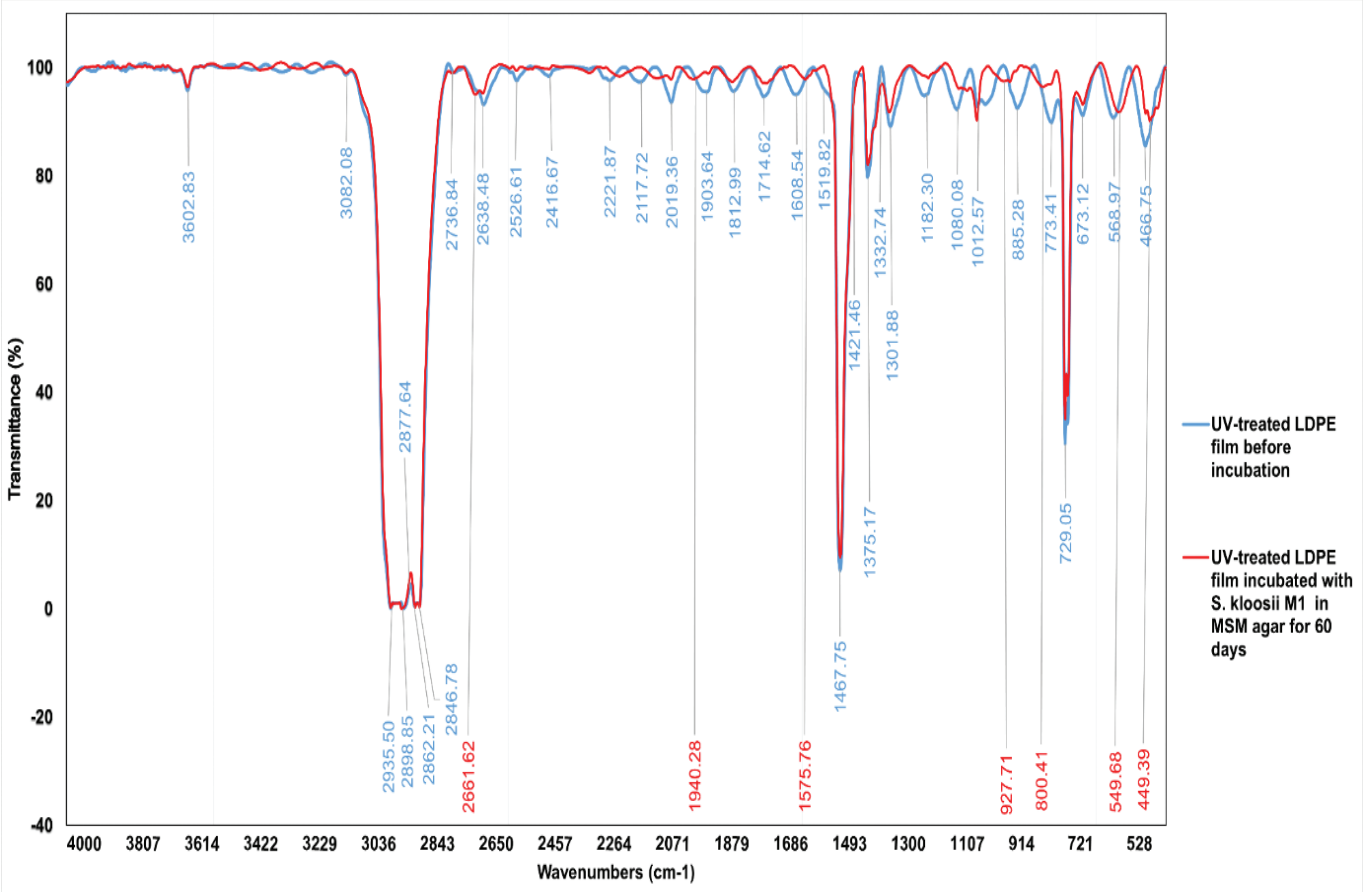

Fig. 4. FT-IR spectra of UV-treated LDPE film before and after incubation with *S. kloosii* M1 in MSM agar for 60 days.

The carbonyl index (CI) is a valuable parameter used to monitor oxidation reactions in polyethylene (PE). It specifically focuses on changes in the carbonyl band (C═O) observed in Fourier transform infrared (FTIR) spectra [29]. In the FT-IR spectral analysis control and degraded LDPE showed absorption intensity at 1467.75 cm^{-1} was 20.65 and 9.91, respectively; and control and degraded LDPE intensity at 1714.62 cm^{-1} were 99.64 and 97.15, respectively. The carbonyl index was evaluated by the following formula [29] - Carbonyl Bond Index (KCBI) = I_{1714}/I_{1467}. The carbonyl index decreased by 22.64%, indicating bacterial cleavage of carbonyl

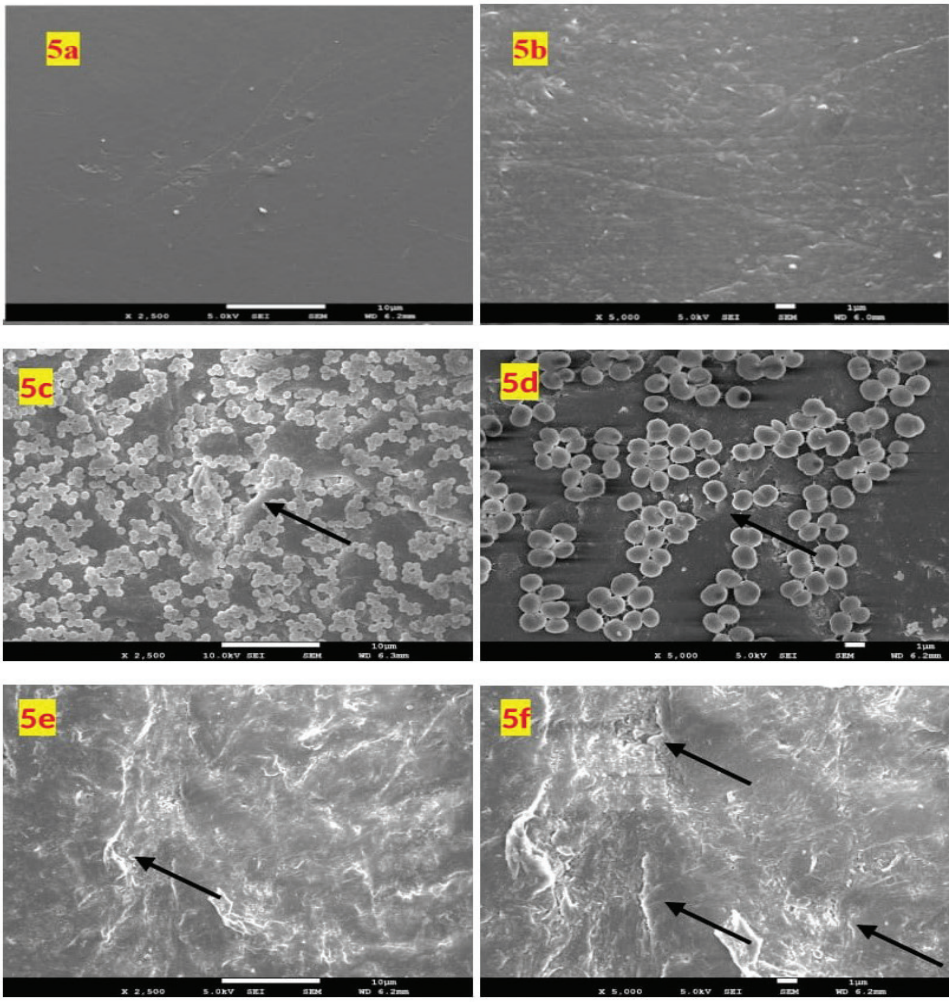

Fig. 5. Scanning electron micrograph of LDPE films. [(From left to right): 5a and 5b depicts control LDPE films at 2500X and 5000X magnifications; 5c and 5d depicts bacterial colonization of *S. kloosii* M1 at 2500X and 5000X magnifications; 5e and 5f depicts surface erosions, cracks, and pit formation after 60 days of incubation with *S. kloosii* M1 at 2500X and 5000X, respectively].

bonds and energy utilization through β oxidation.

Chatterjee and their colleagues observed C-H stretching, CH_2 asymmetric bending and CH_2 rocking at different wavelengths of infrared due to degradation of LDPE by *Staphylococcus epidermidis* strain BP/SU1 after 15 days of exposure [25]. Harshvardhan and Jha also reported that the carbonyl index of the polyethylene increased after incubation with the bacterial isolates, indicating the degradation and modification of the polymer by the enzymatic activity of the bacteria [20]. Kavitha and their colleagues reported a change in intensities of the alkane group of polyethylene explained *Staphylococcus arlettae* is capable of degrading polyethylene [29]. Gupta and co-workers reported an increase in the carbonyl index attributed to the metabolic activity of bacterial biofilm formation [24]. Jain and colleagues reported LDPE degradation by decrease in carbonyl index (58.8%) and change in functional group vibrations due to *Pseudomonas aeruginosa* strain P9 [33].

Surface analysis

LDPE No. 3 was examined for bacterial growth and biofilm formation. Examination of scanning electron micrograph at 2500x and 5000x magnification (Fig. 5 a, b, c, and d) showed colonization of *Staphylococcus kloosii* M1. Notably, the LDPE surface displays signs of surface erosion in the form of cracks in and around bacterial colonies indicative of degradation. In the control group, no bacterial presence was observed, and the LDPE surface remained smooth.

LDPE No. 4 was examined at 2500x and 5000x for surface erosion and compared with control (Fig. 5 e and f) Scanning electron micrograph showed surface

erosions, cracks and pit formation which was indicative of degradation of LDPE film.

Staphylococcus spp., as a biofilm-producing organism, can attach to surfaces, including low-density polyethylene (LDPE). The EPS produced by *Staphylococcus* spp. contains polysaccharides, proteins, and DNA. This matrix helps stabilize the biofilm and facilitates attachment [25]. Biofilm production of *Staphylococcus haemolyticus strain* on LDPE film was successfully characterized by Sarkar and their colleagues [32]. Zhang and their co-workers also reported the degradation of plastic by changes in surface structure including cracks and holes, roughening of the surface, and formation of microbial colonies over the surface [31]. Another study mentioned that *Staphylococcus epidermidis* could survive on LDPE as its only carbon source and degrade it, as seen by SEM [24]. Jain and their colleagues reported biofilm formation, crakes, pits, surface erosions on the LDPE surface after 60 days of incubation with *Psedomonas aeruginosa* strain P9 [33].

CONCLUSION

This study focuses on the biodegradation of LDPE using *Staphylococcus kloosii* M1, isolated from cattle dung. The study found that *Staphylococcus kloosii* M1 bacteria is highly effective in breaking down LDPE, especially when exposed to UV treatment. Moreover, the ability to synthesise biofilm and adhere to the polymeric surface of LDPE film further enhances the degradation process. These results highlight the importance of exploring diverse microbial communities in the search for effective biodegrading microorganisms. The study found that strain M1, isolated from cattle dung, can efficiently use LDPE as a sole carbon source, contributing to its degradation. This is the first report of isolating LDPE-degrading bacteria from cattle dung, which calls for further investigation on cattle gut microbiota for plastic degradation. However, more studies are required for degradation of LDPE with different incubation periods, UV exposure, chemical treatment, use of catalyst are needed to effective and eco-friendly management of LDPE pollution.

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